



T-DNA tagged knockout mutation of rice OsGSK1 an orthologue of Arabidopsis BIN2 with enhanced tolerance to various abiotic stresses

Serry Koh  Sang-Choon Lee  Min-Kyung Kim  Eun Ho Koh 
Sichul Lee  Synheung An  Sunghwa Choe  Seong-Ryong Kim

Received: 5 February 2007 / Accepted: 21 July 2007 / Published online: 10 August 2007
Springer Science+Business Media B.V. 2007

Abstract T-DNA-tagged rice plants were screened similar to the one observed with the gain-of-function BIN1/ under cold- or salt-stress conditions to determine the AtSK21 mutant. This suggests that OsGSK1 might be a genes involved in the molecular mechanism for their functional rice orthologue that serves as a negative regulator of abiotic-stress response. Line 0-165-65 was identified as a major factor of brassinosteroid (BR)-signaling. Therefore, we a salt-responsive line. The gene responsible for this GUS propose that stress-responsive OsGSK1 may have physiological roles in stress signal-transduction pathways and OsGSK1 (*Oryza sativa* glycogen synthase kinase3-like gene 1), a member of the plant GSK3/SHAGGY-like protein kinase genes and an orthologue of Arabidopsis

brassinosteroidinsensitive2 (BIN2), AtSK21 Northern blot analysis showed that OsGSK1 was most highly detected in the developing panicles, suggesting that its expression is developmental stage specific. Knockout (KO) mutants of OsGSK1 showed enhanced tolerance to

cold, heat, salt, and drought stresses when compared with environmental stresses often influence growth and seed non-transgenic segregants (NT). Overexpression of the production. By invoking specific mechanisms, plants can full-length OsGSK1 led to a stunted growth phenotype regulate the expression of stress-related genes at the transcriptional level, so that their products function in a stress and tolerance response (Thomashow 1999, Bray et al. 2000, Shinozaki and Yamaguchi-Shinozaki 2000).

Keywords Abiotic stress BR-signaling GUS OsGSK1 T-DNA-tagged rice

Serry Koh and Sang-Choon Lee are co-First authors.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-007-9213-4) contains supplementary material, which is available to authorized users.

S. Koh · S.-C. Lee · M.-K. Kim · S.-R. Kim (✉)
Department of Life Science, Sogang University, Seoul 121-742, Korea
e-mail: sungkim@sogang.ac.kr

J. H. Koh · S. Choe
Department of Biological Sciences, College of Natural Sciences, Seoul National University, Seoul 151-747, Korea

S. Lee · G. An
National Research Laboratory of Plant Functional Genomics, Department of Life Science, Pohang University of Science and Technology, Pohang 790-784, Korea

To understand the functioning of these responsive genes, many studies have focused on the protein kinases and transcriptional factors related to abiotic stress-signaling pathways. In Arabidopsis transcriptional factors DREBs/ CBFs interacting with the dehydration-responsive element/ C-repeat cis-acting element play an important role by controlling gene expression of the abiotic stress-signal transduction pathways (Thomashow 1999, Shinozaki and Yamaguchi-Shinozaki 2000). These abiotic stress-signaling pathways are also regulated by several kinds of protein kinase, e.g., Ca²⁺-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs). For example, overexpression of the rice CDPK7 confers salt, drought, and cold tolerance through the regulation of the signal (Saijo et al. 2000). Likewise, stress-activated MAPK

(SAMK) is activated by cold and drought stresses (Jonak et al. 1996). Rice is one of the world's most important food plants, and is grown under various environmental conditions. However, large gaps still remain in our understanding of other components in those pathways. Therefore, to improve crop yields of this staple, it is important to examine whether plant hormones, such as auxin, gibberellins, brassinosteroids, and ethylene, including the molecular mechanism for defense and stress adaptation as well as the role of each signal-transduction component.

To identify the biological functioning of these genes, identified Arabidopsis BR11 (BRASSINOSTEROID INSENSITIVE 1), which encodes a BR receptor. Its orthologues have also been found in rice (OsBR1), tomato (MeBR1) (Meyerowitz 2000) and insertional mutagenesis (Errampalli et al. 1991; Jeon et al. 2000). Random insertional mutagenesis by T-DNA is the most common for large-scale analysis (Lee et al. 2003b). This approach involves modified T-DNA, which has insertional elements. The kinase and its function has been analyzed by using a loss-of-function mutant (Yamamuro et al. 2000). Furthermore, advantageous because the inserted elements act as a tag for gene identification, and the reporter gene can be easily located by monitoring the spatial and temporal activity of reporter proteins in diverse plant organs (Alvarado et al. 2004). For example, using the insertional mutagenesis identified yet. Yoo et al. (2006) reported that there are 9 pGA2144, a gene trap vector, with GUS has enabled us to determine the expression pattern of tagged genes at various developmental stages or under specific abiotic-stress conditions by monitoring the activity of the fusion protein. This T-DNA tagged gene is now identified as GSK1 a homologous gene of plant GSK3/SHAGGY-like protein kinase/BIN2, with enhanced tolerance to various abiotic stresses.

The GSK3/SHAGGY-like kinases are multi-functional non-receptor Ser-Thr (S/T) kinases (Jonak and Li and Nam 2002). In animals, they are involved in the determination of cell fate, resulting in the organization of the body plan. In mammals, two enzymes GSK3 α and GSK3 β (for glycogen synthase kinase) are encoded by two genes; they are involved in regulating glycogen metabolism (Orena et al. 2000), and in stabilizing the cytoskeleton (Zumbrunn et al. 2001). In higher plants, the GSK3/SHAGGY protein kinase genes are present as small gene families. They have been characterized from a number of plant species (Pay et al. 1993; Tichtinsky et al. 1998; Jonak et al. 2000). In Arabidopsis 10 genes belong to the GSK3/SHAGGY-like gene family. Genetic and biochemical approaches have demonstrated that different plant GSK3/SHAGGY-like genes function in diverse processes, including hormone-signaling, development, and stress responses. For example, Arabidopsis AtGSK1 and AtGSK12 function in perianth and gynoecium development (Dornelas et al. 2000). Another Arabidopsis AtSK21/BIN2 (Brassinosteroid-insensitive 2) a homologue of GSKs acts as a negative regulator to control steroid-signaling and Nam 2002). The Arabidopsis AtGSK1 is also involved in the signal-transduction pathway for salt-stress responses (Piao et al. 1999; 2001). In wheat, TaGSK1 is a GSK3/SHAGGY-like gene regulated by salt stress (Chen et al. 2003). Nevertheless, the functioning and characterization of rice GSKs are still largely unknown.

Materials and methods

Plant materials and stress treatments

Seed of rice (*Oryza sativa* ssp. Japonica cv. Dongjin) were surface-sterilized and germinated, and the plants were cultured hydroponically in a growth chamber (22°C, 16-h photoperiod) (Lee et al. 2003a). For the seedling stress treatments, seeds were germinated in distilled water for 4 days, then placed in Yoshida solution (Yoshida et al. 1976) for another 4 days in the growth chamber. They were then treated with drought (air-drying on filter paper for up to 24 h), cold (4°C or 12°C), heat (45°C), salt (100 mM or 250 mM NaCl in Yoshida solution), or abscisic acid (100 μ M ABA in Yoshida solution).

Isolation of T-DNA β -banking sequence by inverse PCR (IPCR) and sequence analysis

After samples of mature rice leaves from selected lines were ground in an MM300 Mixer Mill (Retsch, Germany), genomic DNA was extracted according to the method of Chen and Roland (1999). To isolate the T-DNA β -banking

sequence, IPCR was performed as described previously (Jeon et al. 2000) using two pairs of primers: GUS2 and H3IPCR1 (first PCR), and GUS1 and H3IPCR2 (second PCR). Sequences for the primers included:

H3IPCR1: 5'-CGAGACAACGCAGAGAAAG-3',
 GUS2 : 5'-CTGCATATAACCTGCACATTAGC-3',
 H3IPCR2: 5'-TTCGTA CTGCCTCTCTCC-3'
 GUS1 : 5'-GGATACAAGTCTGTACCTTG-3'

Samples were amplified for 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. PCR products were directly sequenced with the appropriate primers. Genomic sequences matching the tagging sequences were

retrieved from the Chinese Rice Genome Database (<http://www.bti.genomics.org.cn/rice/>) (Yu et al. 2002), and were annotated with the Softberry program (<http://www.softberry.com/berry.phtml>) and BLASTP (<http://www.ncbi.nlm.nih.gov/BLAST/>). The deduced amino acid sequences of the family protein were downloaded and aligned by ClustalX (<http://www.igbmc.ustrasbg.fr/Bioinfo>) and the alignments were edited with GeneDoc (<http://www.psc.edu/bomd/genedoc/>) (Nicholas et al. 1997). A phylogenetic tree was constructed with MEGA 3.1 software using the results of multiple alignments.

Southern and northern blot analysis

Leaf genomic DNA was extracted as described by Chen and Roland (1999). Samples (5 µg) were digested with restriction enzymes for 12 h at 37°C, then separated on a 0.8% agarose gel and vacuum-transferred (Hoefer, USA) to a Hybond-N membrane (Amersham, UK). For northern blot analysis, total RNA was isolated with Tri Reagent (Molecular Research Center, USA). Samples (10 µg) were resolved on a 1.3% agarose gel, and blotted onto a nylon membrane (Sambrook et al. 1989). Hybridization of DNA and RNA blots was performed with the radio-labeled OsGSK1 probe, which was prepared with the RT-PCR product that included the ORF of OsGSK1 as probe after the gel-elution. OsGSK1 cDNA was labeled with [γ -³²P] dCTP, using the random priming method (Feinberg and Vogelstein 1983). After hybridization, the membrane was washed sequentially with 2SSC, 0.1% SDS at RT for 15 min; 1-SSC, 0.1% SDS at RT for 15 min; and 0.1SSC, 0.1% SDS at RT for 15 min. Hybridization signals were detected with an image analyzer (BAS-1500, Fuji, Japan) and exposed on Hyperfilm MP (Amersham, UK).

Genotyping for OsGSK1 knockout

PCR was conducted to distinguish the genotypes of the progeny from our T-DNA tagging lines. Reactions were

carried out with 250 ng of genomic DNA as template and 0.5 units of Ex-Taq polymerase (Takara, Japan), over 35 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min 30 s. The forward (P1) and reverse (P2) primers for OsGSK1 were 5'-TTCCTCAGATTAAGGCTCATCC-3' and 5'-GAAGGAAAGAGGGTTCATTGC-3', respectively. Our T-DNA-specific reverse primer (P3) was 5'-CTGCATATAACCTGCACATTAGC-3' on the right border.

Histochemical GUS staining was performed as described by Dai et al. (1996), except that 20% methanol was added to the staining solution. Samples were incubated in a X-Gluc solution according to the method of Jeon et al. (2000). Chlorophyll was removed with 70% and 95% methanol. GUS-stained samples were then examined under a dissecting microscope.

The full ORF of OsGSK1 was amplified using the OsGSK1 forward (5'-ATGGATCCATGGAGGCGCCGCGGGGCC-3') and -reverse primer (5'-CCGCTCGAGTTAGCTCCAGCATGCGCAAAG-3'). RT-PCR products were cloned and confirmed by nucleotide sequencing, then used as probes for Southern and northern hybridizations. For RT-PCR analysis of the OsGSK1 T-DNA knockout (KO), the gene-specific primers OsGSK1 OsGSK1-GUS and downstream genes were also used (Table 1). Primers specific to the rice actin gene *Act1* (McElroy et al. 1990) served as an RT-PCR control. Total RNA (10 µg) was used for the RT-PCR analysis, according to the method of Takakura et al. (2000). PCR was performed in a 25 µl solution containing a 1 µl aliquot of the cDNA mixture at 1/5 dilution, 0.2 µM of gene-specific primers, 10 mM deoxyribonucleotide triphosphates (dNTPs), and 0.5 units of Ex-Taq DNA polymerase (Takara, Japan). Samples were amplified at 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min 30 s. In all, 22–50 PCR cycles were performed for amplification of specific genes.

Analysis of brassinosteroid sensitivity

We germinated seeds from OsGSK1KO, non-transgenic segregant (NT), and wild-type (WT) plants on MS agar plates in the presence or absence of 8 μ M brassinolide (BL), the most biologically active BR. After 3 days, lengths of the seedling coleoptiles were recorded.

Table 1 Primers used in RT-PCR analysis of OsGSK1 knockout mutant

Primer	Nucleotide sequence
OsGSK1forward	5'-TTCCTCAGATTAAGGCTCATCC-3'
OsGSK1reverse	5'-GAAGGAAAGAGGGTTCATTGC-3'
OsGSK1-GUSforward	5'-TTCCTCAGATTAAGGCTCATCC-3'
OsGSK1-GUSreverse	5'-CAG GCA CAG CAC ATC AAA GA-3'
OsXTR3forward	5'-GGGAGAGAAAAGTTCGGTTC-3'
OsXTR3reverse	5'-TATGCCATTAGCTTCCCAAG-3'
OsEXP4forward	5'-CCAGTTCTAGCCGCCACCGACATC-3'
OsEXP4reverse	5'-ATTCCG TTG CAAGGCCATCACTCC-3'
SalT-forward	5'-TAAGCGACCACGAAGAGTATA-3'
SalT-reverse	5'-AGTGATACCAATATGAGAAACACATAA-3'
RAc1forward	5'-CATGCTATCCCTCGTCTCGACCT-3'
RAc1reverse	5'-CGCACTTCATGATGGAGTTGTAT-3'

Analysis of stress tolerance

Chlorophyll fluorescence was measured with a Plant Efficiency Analyzer (Hansatech, UK) according to the method of Jeong et al. (2002), with minor modification. Approximately 5-cm-long segments of the youngest leaves from 8-day-old plants were air-dried for 6 h in a PEA clip in a 29 °C incubator (70% RH, 60 μmol m⁻² s⁻¹ irradiation from continuous light). Fluorescence signals from samples that were first dark-adapted for 30 min were then measured over various time periods. The ratio of Fv to Fm (Fv/Fm), representing the activity of Photosystem II, was used to assess functional damage to the plants (Genty et al. 1989). To test their survival after cold stress treatments, 8-day-old seedlings were cultured in Yoshida solution and treated with 4 °C for 4 days. They were then allowed to recover under normal growing conditions for 7 days. To apply heat stress, 8-day-old seedlings cultured with Yoshida solution were exposed for 2 h at 45 °C, as described by Katiyar-Agarwal et al. (2003). After a recovery period, wilted and healthy plants were counted. To test salt tolerance, 8-day-old seedlings were cultured in Yoshida solution and treated with 100 mM NaCl for 7 days and 250 mM NaCl for 2 days, respectively. They were then recovered under normal growing conditions for 7 days and checked for wilting ratio.

Generation of transgenic Arabidopsis plants

To generate expression constructs of OsGSK1 gene, a set of primers which was designed to amplify the full length cDNA was used: GSK1-FL-F-5'-TCTGGGAGCC TCGAGCGG-3', GSK1-FL-R-3'-TGAAACGCGTACGACCCTCGGAGCTCGCC-5'. The forward (F) primer is flanked with a restriction enzyme site for EcoRI and the reverse primers with the Xhd site. The amplified DNAs

and the pENTR Gateway cloning vector (Invitrogen, USA) were digested with the same restriction enzymes before being ligated with each other to result in a construct named pSK305, which harbors full length cDNA. The two entry clones were subjected to LR cloning with pMDC139 destination vector (Curtis and Grossniklaus 2003), and named OsGSK1_{III}. The cloned DNA was introduced into an Agrobacterium GV3101 strain before being introduced into Arabidopsis thaliana Columbia wild type by using the spray transformation technique (Choe et al. 2001). Thirty primary transformants were isolated based on their resistance to the antibiotics Hygromycin (50 mg/ml). Ten representative lines were selected and further screened until T₂ generation to obtain a homozygous line.

Results

Identification of rice GSK3/SHAGGY homologous gene, OsGSK1

We have previously reported the generation of T-DNA insertional mutant lines with pGA2144 (Jeon et al. 2000). The tagging vector pGA2144 contains the promoter-less gene that can generate a fusion between the reporter and tagged gene. We screened the population for stress-inducible genes by GUS-staining after cold or salt treatment. A salt-responsive line, 0-165-65 was identified from this screening, and the T-DNA-tagged gene was determined by obtaining the flanking sequence via IPCR. This flanking sequence was used to search the National Center for Biotechnology Information (NCBI) database. The tagged gene was an unnamed protein (BAA92966) with 96% similarity to Arabidopsis GSK3/SHAGGY-like kinase 21 (CAA64409) at the amino acid level. We named this gene OsGSK1 (*Oryza sativa* glycogen synthase kinase3-like gene1), based on the high similarity of the deduced protein

with other GSK proteins OsGSK1 (Os01g10840), located on Chromosome 1, was composed of 12 exons, and had 404 amino acid residues. Arabidopsis dwf12 mutants, which are allelic with 1.2-kb cDNA that encoded 404 amino acid residues. Ubin2 and ucu1 mutants, are all mis-sense mutations in the inverse PCR results revealed that the T-DNA was inserted into a TREE domain that alter kinase activity (Choe et al. 2002, Li and Nam 2002, Perez-Perez et al. 2002). Based on our comparisons of GSK sequences, a phylogenetic tree was developed. Plant GSKs are divided into four subgroups (Charrier et al. 2002, Yoo et al. 2006). Here, OsGSK1 was classified into Subgroup II with AtSK21, AtSK22, AtSK23, and OsSKgamma (Fig. 3). Among those members, OsGSK1 had a highly conserved kinase domain, and the highest similarity with AtSK21, which is known as a negative regulator of brassinosteroid-signaling, BIN2 (Li and Nam 2002, Yoo et al. 2006). That TREE domain is highly conserved among Arabidopsis GSKs and likely plays a critical role in the functioning of

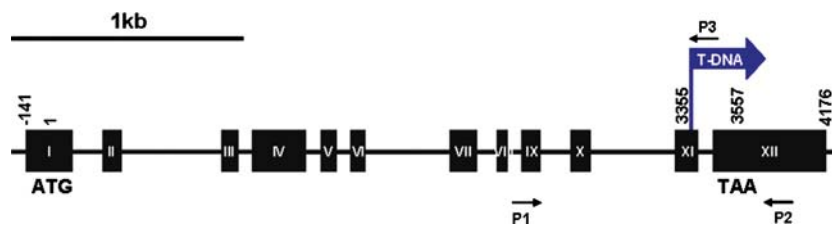


Fig. 1 Schematic diagram for T-DNA insertion site of OsGSK1 and its orientation; small arrows, gene-specific primers for RT-PCR analysis and genotyping of tagged gene. ATG and TAA lines between boxes are introns. Large arrow indicates T-DNA insertion start and stop codon. Scale bar = 1 kb

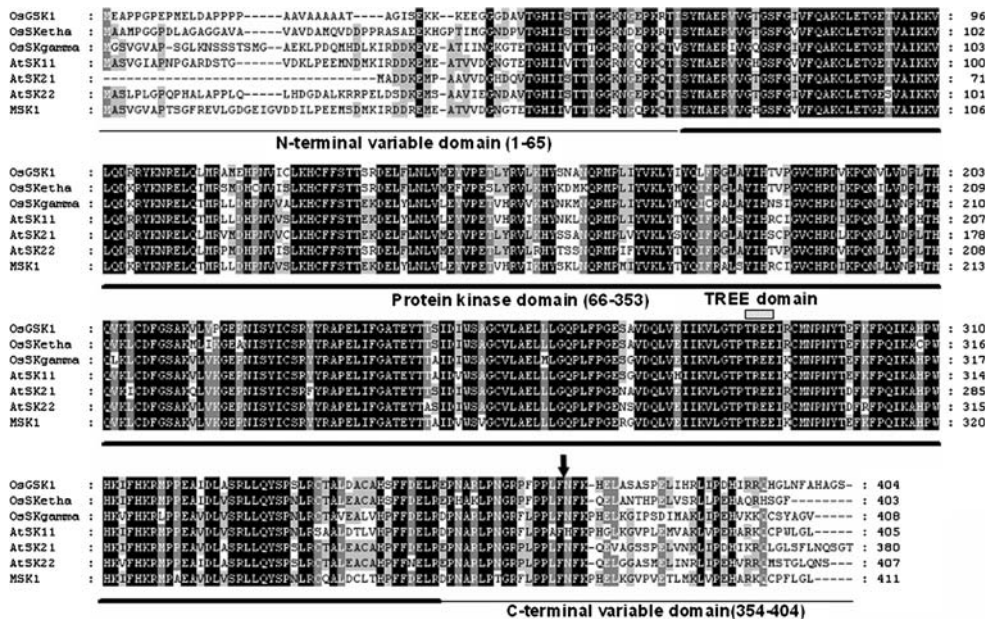


Fig. 2 Sequence alignment of amino acid sequences of OsGSK1 with other plant GSKs homologues. Deduced amino acid sequence of Arabidopsis OsGSK1 was aligned with sequences obtained from public databases: Arabidopsis AtSK11 (X75432), AtSK21 (X94939), and AtSK22 (X99696); Medicago sativa MSK1 (X68411); Oryza sativa OsSKgamma (AB59612). A vertical arrow indicates a putative Thr phosphorylation site by casein kinase II. Thin bars delimit N- or C-terminal variable domain; thick bar represents the T-DNA insertion site

Expression analysis of OsGSK1

To determine the expression pattern of OsGSK1 we isolated total RNA from the calli, whole seedlings, mature leaves, panicles, and developing seeds of wild-type plants and used these materials for northern blot analysis. OsGSK1 transcript was detected, at varying levels, in all these tissues or organs except for mature leaves (Fig. 4). Expression was highest in the young panicles, with slightly less detection in the calli, implying possible physiological role of OsGSK1 in developing tissues.

We also performed northern blot analysis to investigate OsGSK1 expression under stress conditions (Fig. 5). Following cold treatment, transcript levels reached a maximum at 3 h, then decreased. Under salt stress, transcripts slightly increased. Drought stress caused a gradual decrease in transcript levels over 24 h. Under ABA treatment, expression was not significantly different from that detected in our control. These results indicate that OsGSK1 transcript accumulations are regulated differently by environmental stresses.

Southern blot analysis of OsGSK1

To investigate the number of OsGSK1 homologous genes in the rice genome, Southern blot analysis was performed using OsGSK1 as probe. Four hybridizing bands were observed in each lane (data not shown). Because the genomic DNA sequence (11971.t00957) of OsGSK1 contained 1 EcoRI, 1 BamHI, and 2 HindIII sites, the additional bands found on this blot implied the existence of another homologous gene with high homology to OsGSK1. To confirm this possibility, we searched the TIGR rice database with the BlastN algorithm, using the ORF sequences of OsGSK1. Another homologous gene, locus Os05g11730, was identified, with 91.5% homology to OsGSK1 in the ORF sequence. Phylogenetic study of plant GSK3/SHAGGY-like kinase by Yoo et al. (2006) revealed nine GSK3/SHAGGY-like kinase homologues in rice, including OsGSK1.

Identification of OsGSK1 knockout (KO) mutant

To identify the OsGSK1 knockout (KO) mutants, 100 plants of Line 0-165-65 were grown in soil and genotyped by PCR. From 11 progeny, those numbered 1, 6, 7, 8, 9, and 10 were determined to be T-DNA homozygotes, meaning that these progenies were OsGSK1 KO mutants (Fig. 5A and B). Among the remaining progenies, Numbers 2, 3, and 11 were T-DNA heterozygotes (Ht) and Numbers 4 and 5 were non-transgenic segregants (NT). We conducted GUS assays with 8-day-old KO mutants to investigate the expression pattern of OsGSK1 GUS

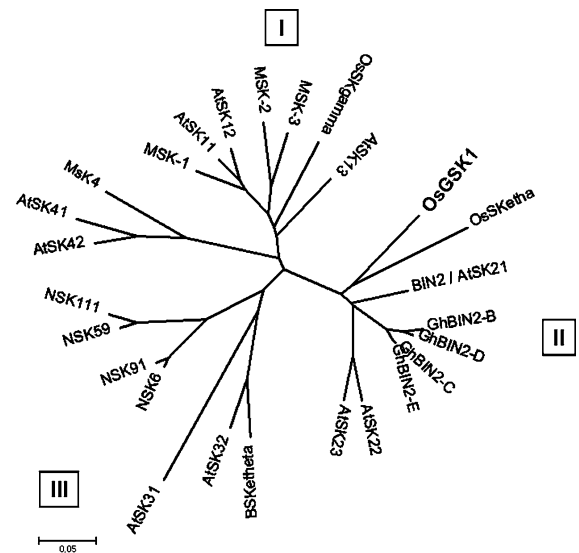


Fig. 3 Phylogenetic tree of OsGSK1 with other plant GSKs including BIN2-homologues. GSKs family is divided into 4 subgroups, and OsGSK1 belongs to Group II. Tree was generated by ClustalW and neighbor-joining algorithm in MEGA 3.1 software. Arabidopsis thaliana: AtSK11(X75432), AtSK12(X75431), AtSK13(AL163792), BIN2/AtSK21 (X94939), AtSK22 (X94938), AtSK23 (X99696), AtSK31 (AJ002280), AtSK32 (Y07822), AtSK41 (X79279), and AtSK42(AC079732); Brassica napus BSKtheta(Y12674); Gossypium hirsutum GhBIN2-B, GhBIN2-C, GhBIN2-D, and GhBIN2-E (Sun and Allen 2009); Medicago sativa MSK1 (X68411), MSK2 (X68410), MSK3 (X68409), and MSK4 (AF432225); Nicotiana glauca NSK6 (Y08607), NSK59(AJ002315), NSK91 (AJ224163), and NSK111(AJ002314); Oryza sativa OsSKgamma (AB59612) and OsSKtheta(Y13437).

Furthermore, both T-DNA homozygote and heterozygote plants showed positive GUS activity, while the NT plants exhibited none. This indicated that the T-DNA insertion of OsGSK1 co-segregated with GUS activity. To confirm whether these activities originated from the OsGSK1-gus fusion gene, we analyzed expression of OsGSK1 and no. Os05g11730, was identified, with 91.5% homology to OsGSK1 in the ORF sequence. Phylogenetic study of plant GSK3/SHAGGY-like kinase by Yoo et al. (2006) revealed nine GSK3/SHAGGY-like kinase homologues in rice, including OsGSK1. Furthermore, nucleotide sequence analysis of the OsGSK1-gus fusion transcript showed that the gus gene was in-frame-fused to the OsGSK1 transcript with intra-sequences from T-DNA (data not shown). These results demonstrated that positive GUS activity in the KO mutant resulted from OsGSK1-gus fusion by T-DNA insertion.

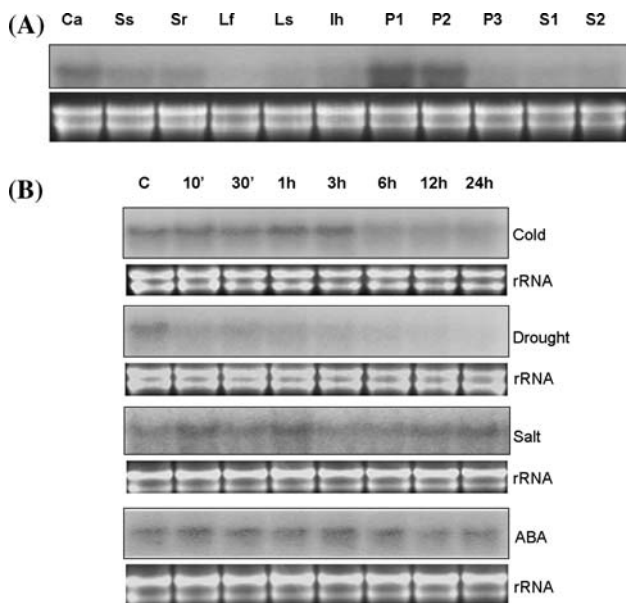


Fig. 4 Northern blot analyses of *OsGSK1* throughout developmental stages. **A**) Total RNA (30 μ g) from callus (Ca), 7-day-old shoots (Ss), 7-day-old roots (Sr), mature leaves (Lf), flag leaf sheaths (Ls), highest internodes at pre-heading stage (lh), 1- to 2-cm-long panicle (P1), 3- to 8-cm-long panicles (P2), mature panicles (P3), developing seeds at 3 DAP (S1), and 6 DAP (S2), was separated, blotted, and hybridized with *OsGSK1* as probe. EtBr-stained rRNA bands indicate amount of RNA loading. **B**) Total RNA from stress-treated seedlings for indicated time. C, Control; cold, C; drought, air-drying; salt, 250 mM NaCl; ABA, 100 μ M ABA. Total RNA (30 μ g) was used and hybridized with *OsGSK1* as probe. EtBr-stained rRNA bands indicate amount of RNA loading

activity was strong in the root tips and root hairs but was more weakly detected in the shoots (Fig. 4A). In the latter, activity was localized to the lamina joint in the collar region. GUS activity also was detected in the vascular bundles of the coleoptile (Fig. 6). In contrast to *OsGSK1* KO, non-transgenic (NT) plants exhibited no GUS activity (data not shown). At the flowering stage, activity was highly detected in the entire young panicle (Fig. 4D). In the spikelet, GUS activity was found in the awn and vascular bundles of the palea and lemma (Fig. 6). Whereas activity was strong in the stigma and rachilla, it was barely detected in the anther (Fig. 4C).

Until the flowering stage, no morphological alterations were observed in the KO plants. However, at that stage, the shape of the spikelets from those mutants differed from those of NT plants. Likewise, the *OsGSK1* KO mutant plants had longer awns than did those from the NT or WT. Awn lengths from KO and NT plants were 16.4 ± 3.89 mm and 0.75 ± 0.35 mm, respectively. The de-husked seed weights were 27.68 ± 3.02 mg (KO) and 24.80 ± 1.40 mg (NT), indicating that spikelets were slightly larger on KO plants. However, the level

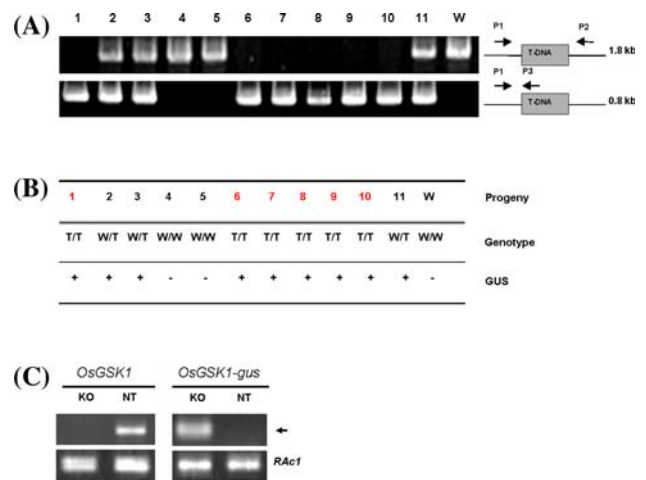
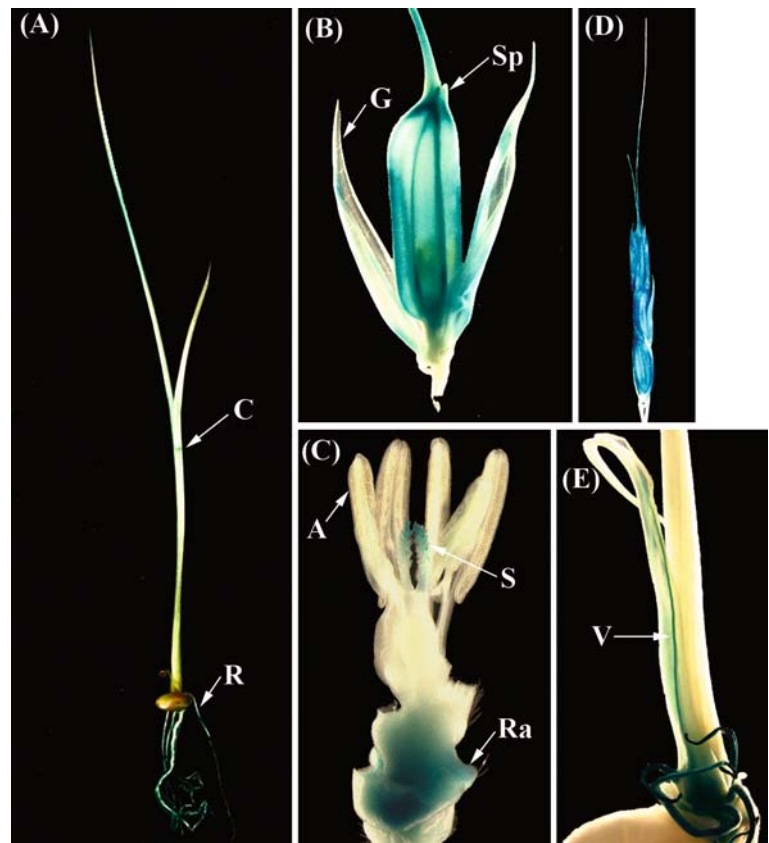


Fig. 5 Genotyping of progeny from Line 0-165-65 for knockout (KO) mutant selection. **A**) Progeny numbered 1–11 were genotyped by PCR using gene- and T-DNA-specific primers. Upper panel indicates non T-DNA insertion; lower panel, T-DNA insertion. **B**) Comparison of genotype with results of GUS assay. **C**) *OsGSK1* expression was investigated in KO mutants (KO; Progeny 1 and 6D10) and non-transgenic segregant (NT; Progeny 4 and 5) by amplification of *OsGSK1-GUS* fusion and natural *OsGSK1* transcript. Rice actin gene, *Rac1*, was used for PCR control. T/T, T-DNA homozygote; W/T, T-DNA heterozygote; W/W, wild type; +, positive GUS activity; -, non-GUS activity; W, wild type

BR sensitivity and BR-responsive genes in *OsGSK1* KO mutant

To investigate the involvement of *OsGSK1* in BR-signaling, we evaluated BR-sensitivity in the *OsGSK1* KO mutant by measuring coleoptile lengths and checked for the expression patterns of BR responsive genes in KO mutant after BR treatment. When seeds were germinated on MS agar media containing 10⁻⁶ M brassinolide (BL), the KO seedlings showed more coleoptile elongation compared with the NT (Fig. 7A) (9.0 ± 1.2 mm (KO) versus 6.6 ± 0.5 mm (NT) (Fig. 7B)). We also examined the expression of *OsXTR3*, *OsEXP4* and *SalT* in the KO mutant, performing RT-PCR after treatment with 10⁻⁶ M BL (Fig. 7C). *OsXTR3* encodes xyloglucan endotransglycosylase/hydrolase (XTH), a cell wall-modifying enzyme essential for cell elongation, and for which expression is up-regulated by BR (Uozu et al. 2000). *OsEXP4* encodes alpha-expansin, which is important to stem elongation and induced by submergence and GA (Cho and Kennerly 1997). *SalT* encodes a lectin-like protein, and is up-regulated by various abiotic stresses and BRs (Supplemental Fig. 1). In our experiments *OsXTR3* expression reached a maximum at 12 h, then slightly decreased in both KO and NT plants. *OsXTR3* transcript in the KO mutant was strong at each treatment point compared with

Fig. 6 Histochemical localization of GUS activity and morphological alteration of *OsGSK1*KO mutant. (A) 7-day-old seedlings. C, collar region; R, root. (B) Floret. G, glume; Sp, spikelet. (C) Inner portion of spikelet in (B). A, anther; S, stigma; r, rachilla. (D) Young panicle. (E) Close-up of coleoptile region from seedling in (A). V, vascular bundle of coleoptile



the NT plants. Expression of *OsEXP4* in the KO was slightly higher than in the NT for up to 12 h after BL treatment. *OsEXP4* expression was similar to that of *OsEXP4* in the NT plants. When we combined these results with those from our coleoptile-elongation examination, we could conclude that the KO mutant was more sensitive to BL treatment, both physiologically and as indicated by BR-responsive gene expression.

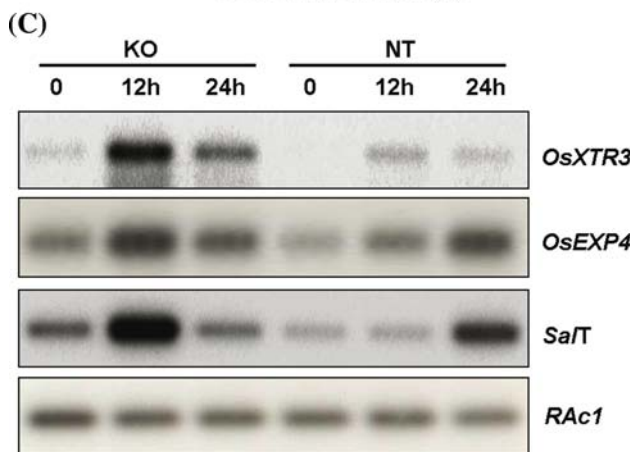
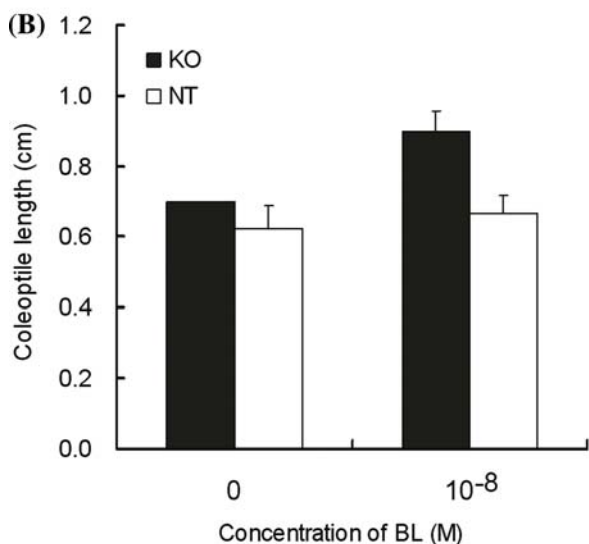
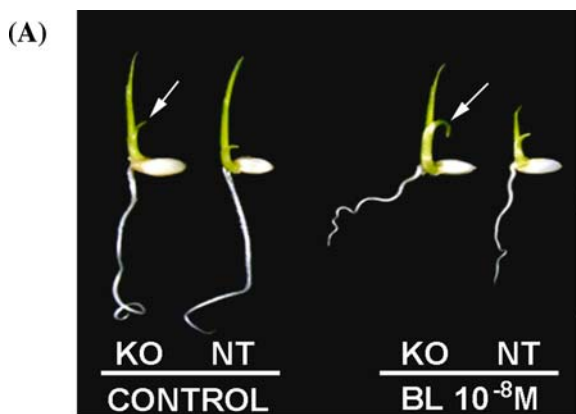
Dwarfism caused by ectopic overexpression of *OsGSK1* gene in Arabidopsis

To determine whether *OsGSK1* acts as a negative regulator (such as does Arabidopsis *BIN2/DWF1*) in BR signal-transduction pathways (Choe et al. 2002, Li and Nam 2002), we also generated transgenic plants that ectopically over-expressed full length *OsGSK1* gene. Relative to the vector control (Fig. 8), the overall height of the 35S::*OsGSK1*_{full} plants reached only 1/3 the height of the vector control. Although the 35S::*OsGSK1*_{full} transgenic plants did not show dwarfism, this stunted growth phenotype suggested that *OsGSK1* negatively controlled the growth of Arabidopsis plants. This phenotype has also been seen when the Arabidopsis *BIN2/DWF1* gene is overexpressed

(Li and Nam 2002). Furthermore, it has been shown that BR deficient dwarf mutants of Arabidopsis display slightly late-flowering phenotype in that the inflorescences and floral organ develop slower relative to wild type control (Kwon and Choe 2005). Therefore, it is likely that *OsGSK1* functions as a negative regulator in BR signal-transduction pathways.

Stress tolerance in *OsGSK1*KO mutants

To examine the role of *OsGSK1* in the abiotic-stress response, we analyzed the tolerance of *OsGSK1* KO mutants by measuring chlorophyll fluorescence and the wilting ratio after 8-day-old seedlings were treated with drought, cold, salt, or heat (Fig. 9 and Table 2). Compared with our NT plants, the wilting ratios for KO mutants were about 20% lower after cold stress (Fig. 9A) and as much as 26% lower after heat stress (Fig. 9C). The wilting ratios for KO were lower each 13% and 36% than NT plants by 100 mM and 250 mM NaCl treatment, respectively (Fig. 9B, C). For our drought-tolerance test, leaves from hydroponically grown 8-day-old KO and NT seedlings were air-dried on filter paper. Under normal growing conditions, the ratio



◀Fig. 7 Analysis of BR sensitivity and BR-responsive genes in OsGSK1KO mutant. A) Seeds of OsGSK1KO, NT, and WT were germinated on MS agar plates in presence or absence of BL. After 3 days, seedling morphology was investigated. B) Effect of BL on coleoptile length in OsGSK1KO and NT was investigated after germination under same conditions as in (A), with indicated BL concentration. C) RT-PCR analysis was conducted with cDNA from OsGSK1KO and NT seedlings after treatment with 10⁻⁸M BL, using gene-specific primers for OsXTR3, OsEXP4 and SaIT. Rice actin gene, RAc1, was used for PCR control. KO, T-DNA homozygote; NT, non-transgenic segregant; WT, wild type

Expression analysis of abiotic stress-responsive genes in OsGSK1KO mutant

We analyzed the expression of several stress-responsive genes in the KO mutants to determine why OsGSK1KO showed enhanced abiotic stress tolerance. Previously reported stress-responsive genes OsNT (Z25811), lip5 (AB011368), and OsDhn1 (AY786415) were used for our comparison of KO and NT plants by northern blot analysis (Fig. 10). Under normal growing conditions (control), expression did not differ between the two genotypes. However, up-regulation of SaIT by salt treatment was 1.8-fold greater in KO than in NT. In contrast, neither drought nor ABA changed SaIT expression in the KO mutants. lip5 was expressed more in KO than in NT as a result of salt, drought, or ABA treatment, and OsDhn1 was more highly expressed in KO, particularly in response to drought stress. Overall, these stress-responsive genes were highly induced in KO plants under different abiotic-stress conditions.

Discussion

We have now identified the stress-responsive gene OsGSK1 in rice, using T-DNA insertional mutagenesis, and have characterized its physiological role in response to abiotic stresses. In higher plants, the GSK3/SHAGGY-like kinases are a small family of Ser/Thr protein kinases that function in various developmental processes, e.g., hormone/stress-signaling and floral development (Dornelas et al. 1998; Piao et al. 1999; Li and Nam 2002; Chen et al. 2003; Yoo et al. 2006). The T-DNA insertional position of OsGSK1 is at the 11th exon, which is in the C-terminal region of the OsGSK1 protein (Fig. 1). This position corresponds to 35 amino acid residues from the C-terminal end of OsGSK1 protein, and is external to the functionally important region of GSKs known as the TREE domain, which is inside a conserved kinase domain (Choe et al. 2002). Nine homologues of GSK3/SHAGGY-like genes exist in rice (Yoo et al. 2006). OsGSK1 belongs to Subgroup II, which shows higher similarity to Arabidopsis

of Fv to Fm was 0.82 ± 0.01 for both KO and NT plants (Fig. 9D). However, changes in Fv/Fm values varied between these genotypes, showing a slower decline in the KO mutants. After 6 h of drought treatment, the KO ratio was reduced to 0.47 ± 0.15, which was >2-fold higher than that of NT plants. These data indicated that the OsGSK1 KO mutant had enhanced tolerance to drought stress.



Fig. 8 Morphological effects caused by ectopic overexpression of OsGSK1 in Arabidopsis. Two transgenic plants (left to right) harbor pMDC139 empty vector as control and 35S::OsGSK1_{full}, respectively. Compared with vector control, overall heights of 35S::OsGSK1_{full} plants were only 1/3 that measured from the vector control. Scale bar = 3 cm

(BIN2) encoding AtSK21 (Li and Nam 2002) shares the highest sequence similarity to OsGSK1 (Fig. 2). The relationship between brassinosteroid-signaling and OsGSK1 is further discussed below.

Because of its high expression in the developing panicles, we can suggest that OsGSK1 is involved in floral development (Figs 4A and 6). This hypothesis is further supported by our finding that OsGSK1KO plants have longer awns and slightly larger spikelets than do the NT plants. Our stress-tolerance test also demonstrated that the OsGSK1KO plants have significantly enhanced tolerance against drought, heat and cold temperatures (Fig. Table 2). Therefore, we propose that OsGSK1 functions as a negative regulator in the general abiotic-stress response in rice. If true, we would expect the expression level of stress-responsive genes to be up-regulated in KO plants. In fact, SalT is regulated by abiotic stresses, including those from cold, salt, drought, and the phytohormones jasmonic acid, ABA, and salicylic acid (Claes et al. 1990; Rabbani et al. 2003; Kim et al. 2004).

GSK3/SHAGGY-like genes AtSK21, AtSK22, and AtSK23 (Fig. 3). The Arabidopsis genome contains 10 GSK3/SHAGGY-like genes, and Brassinosteroid Insensitive 2

In the current study, three abiotic stress-responsive genes SalT, lip5, and OsDhn1 showed altered expression in OsGSK1KO plants (Fig. 10). Thus, we conclude

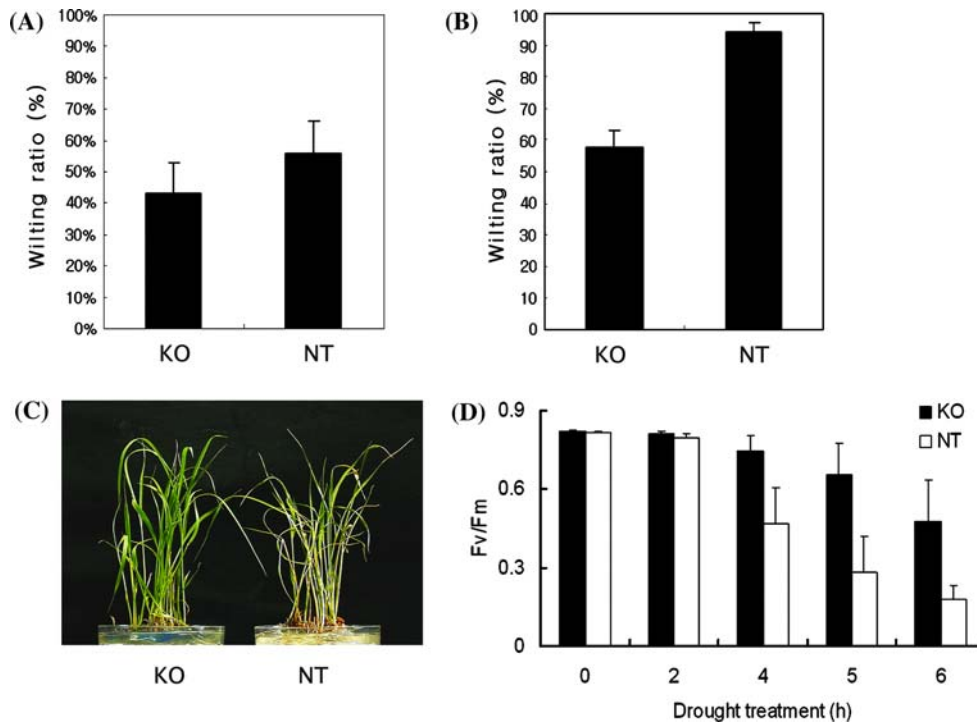


Fig. 9 Salt and drought tolerance tests of OsGSK1KO mutant. (A) Wilting ratio of OsGSK1KO and NT plants were measured after 7 days of 100 mM NaCl treatment, immediately followed by 7 days recovery period. (B) Wilting ratio of OsGSK1KO and NT plants were measured after 2 days of 250 mM NaCl treatment, followed by 7 days recovery period. (C) After salt stress treatment, OsGSK1KO plants showed higher survival rate than NT plants. (D) Drought-

tolerance test of OsGSK1KO mutant. Chlorophyll fluorescence in OsGSK1KO mutant after drought treatment was measured by Fv/Fm value. Changes in fluorescence in youngest leaves were measured during indicated times of drought treatment. Error bars indicate standard deviation from triplicate experiments. KO, T-DNA homozygote; NT, non-transgenic segregant

Table 2 Wilting ratios for OsGSK1 knockout and non-transgenic plants after stress treatments

Treatment	KO	NT
Cold ^a	43.9 ^c	63.9 ^c
Heat ^b	56.3 ^c	82.4 ^c

^a Cold (4 °C) treatment for 4 days, followed by recovery under normal conditions for 7 days

^b 45 °C treatment for 2 h, followed by recovery under normal conditions for 7 days

^c Percent of plants on which entire leaves were wilted divided by total number of plants tested. KO and NT indicate knockout and non-transgenic segregant OsGSK1-T-DNA-inserted mutant Line 0-165-65

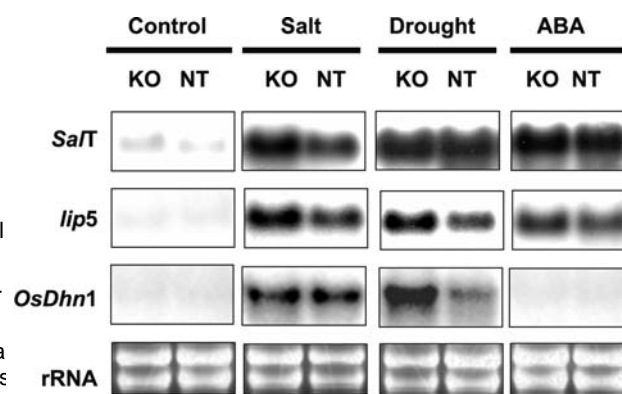


Fig. 10 Northern blot analysis of *SaIT*, *lip5*, and *OsDhn1* in OsGSK1 KO and NT plants following stress. Each lane was loaded with 10 µg of total RNA isolated from 8-day-old seedlings that were treated with 100 µM ABA, 250 mM NaCl, or air-drying (drought) for 12 h. EtBr-stained rRNA bands indicate amount of RNA loading. KO, T-DNA homozygote; NT, non-transgenic segregant

that phenotypic changes to the spikelet at the flowering stage, as well as altered expression levels for stress-responsive genes in those plants, are due to disruptions in their OsGSK1 functioning. Interestingly though, expression of those genes was not significantly different when the KO plants were treated with ABA (Fig. 10). This is somewhat consistent with our northern blot data (Fig. 10) that also showed no change in OsGSK1 expression by ABA. Therefore, these results suggest that the stress-responsive expression of OsGSK1 might be regulated in an ABA-independent manner. In Arabidopsis, stress signals are transmitted by both ABA-independent and ABA-dependent pathways (Bray et al. 2000; Shinozaki and Yamaguchi-Shinozaki 2000; Thomashow 1999). In the former, the DREB2 protein (a transcription factor) is a key component by which osmotic stress-responsive gene expression is modulated (Liu et al. 1998). Likewise, the stress-inducible expression of OsDREB2A (AF300971), a rice homolog of DREB2, is regulated through post-translational modifications, such as phosphorylation and dephosphorylation (Dubouzet et al. 2003). However, protein kinase-interacting OsDREB2A and the gene responsible for its regulation have not yet been found. Whether OsGSK1 is directly connected with these phosphorylation processes or stress-signaling involving the DREB2 protein would be an interesting subject to investigate.

As mentioned above, OsGSK1 has the highest amino acid sequence homology with Arabidopsis BIN2/AtSK21, a negative regulator of BR-signaling (Li and Nam 2002). BRs are steroidal hormones ubiquitously distributed in plant species. BRs play an important role in various cellular responses, e.g., stem elongation, pollen tube growth, xylem differentiation, leaf epinasty, and root inhibition (Mandava 1988; Clouse and Sasse 1998). Like OsGSK1, the cotton BIN2-homologous genes GhBIN2-C and GhBIN2-E are highly expressed in the immature ovules after anthesis (Sun and Alle 2005). However, when

OsGSK1 and OsBR11 are compared, their expressed regions differ, with OsBR11 transcripts being more highly detected in the shoot apex than in the developing flower (Yamamuro et al. 2000). Like the Arabidopsis loss-of-function mutant *bin2-3*, *bin2-3* is generated by a T-DNA insertion into the exon (Vert and Chory 2006). This *bin2-3* mutant shows hypersensitivity to exogenous BL treatment, but retains a normal morphology. Similar hypersensitivity was observed with our OsGSK1KO mutant (Fig. 7A). Therefore, we suggest that OsGSK1 is an orthologue of BIN2, acting as a negative regulator of BR-signaling of rice. However, because rice has at least four BIN2-homologues in Group II (Yoo et al. 2006) and because their functions may overlap, further studies, such as kinase assays and crosses with the OsBR11 mutant, are needed to verify OsGSK1 as a rice BIN2 (OsBIN2). Another potential role for BRs is to increase plant tolerance to various environmental stresses, including low and high temperatures, drought, and salt (Krisnan et al. 2003; Kagale et al. 2007). Kagale et al. (2007) reported the molecular evidence confirming the role of BR in drought, cold, and salt stresses using marker genes with known response to those stresses in both *A. thaliana* and *Brassica napus*. Among these marker genes, the *ERD9A*, *ERD10* and *rd22* genes encode a class of proteins with molecular chaperone-like functions, preventing protein aggregation during drought stress. Accumulations of their transcripts under drought stress at early time point was consistently detected in BR-treated plants (Goyal et al. 2005; Kagale et al. 2007). To investigate the relationship between OsGSK1 and BR-signaling, we conducted a BR sensitivity test by growing young seedlings in the presence of 10

brassinolide (BL) (Fig. 7). KO coleoptiles were longer than those from NT plants, suggesting a BL-response in the former. In addition, we examined the expression patterns of two BR-responsive genes, *OsXTR3* and *SaIT*, in the KO and NT plants (Fig. 7). *OsXTR3* encodes xyloglucan endotransglycosylase/hydrolase (XTH), a cell wall-modifying enzyme essential for cell elongation, and which is up-regulated by BR (Uozu et al. 2000). We also found that *SaIT* was induced by BL exposure (Supplementary Fig. 1), as well as by other abiotic treatments (Claes et al. 1990; Rabbani et al. 2003). Another cell wall-loosening gene, *OsEXP4* was included in the BL treatment (Cho and Kende 1997). In these tests, expression of *OsXTR3* was clearly enhanced in KO plants treated with 10⁻⁶ M BL (Fig. 7C). *OsEXP4* also was up-regulated in the KO plants (Fig. 7C), but not to the same extent as with *OsXTR3*. Therefore, it is apparent that hypersensitivity to BL in the *OsGSK1*KO mutant is due to the reduced activity of *OsGSK1* as a negative regulator by T-DNA insertion (Fig. 7A, B).

Compared to the phenotypes associated with expression of our vector control and a nonfunctional, truncated gene, the ectopic overexpression of *OsGSK1*_{full} in *Arabidopsis* results in stunted growth. This suggests that *OsGSK1* might function as a negative regulator in BR signal-transduction pathways, as also reported in *Arabidopsis* (Li and Nam 2002). Taken together, the phenotype of increased tolerance to stress in KO plants is likely due to elevated activity in those pathways, which is caused by disruption of the gene that encodes for a negative regulator of BR signal transduction in rice. However, because all phenotypes of increased tolerance to stress in KO plants were only observed in a single allele, there was a concern that some of the phenotypes might be caused by mutation at a locus other than *OsGSK1*. Transgenic rice often contains mutation caused by retrotransposons, independent of the T-DNA. A study of allelic lines of *OsGSK1* and functional complementation of *OsGSK1* to KO plants would provide the complete understanding of the function of *OsGSK1*.

Future research should focus on elucidating the mechanism involved with these increased BR-signaling activities, which leads to the induction of genes participating in those stress signal-transduction pathways. This should help us devise approaches that use molecular engineering to confer increased stress tolerance in rice plants. The goal will be to obtain novel crop plants with increased sustainability.

Acknowledgments We thank Priscilla Licht for critical proofreading of the manuscript. This work was funded in part by grants from the Biogreen 21 Program, Rural Development Administration and from the National Research Laboratory Program.

- References**
- Alvarado MC, Zsigmond LM, Kovacs I, Cseplo A, Koncz C, Szabados LM (2004) Gene trapping with preßy luciferase in *Arabidopsis* tagging of stress-responsive genes. *Plant Physiol* 134:18D27
- Bray EA, Bailey-Serres J, Weretilnyk E (2000) Response to abiotic stresses. In: Buchanan BB, Gruissem W, Jones RL (eds) *Biochemistry and molecular biology of plants*. American Society of Plant Physiologists Press, Rockville, MD, pp 1158D1203
- Charrier B, Champion A, Henry Y, Kreis M (2002) Expression profiling of the whole *Arabidopsis* shaggy-like kinase multigene family by real-time reverse transcriptase-polymerase chain reaction. *Plant Physiol* 130:577D590
- Chen DH, Roland PC (1999) A rapid DNA miniprep method suitable for AFLP and other PCR applications. *Plant Mol Biol Rep* 17:53D57
- Chen GP, Ma WS, Huang ZJ, Xu T, Xue YB, Shen YZ (2003) Isolation and characterization of *OsGSK1* involved in wheat salt tolerance. *Plant Sci* 165:1369D1375
- Cho HT, Kende H (1997) Expression of expansin genes is correlated with growth in deepwater rice. *Plant Cell* 9:1661D1671
- Choe S, Fujioka S, Noguchi T, Takatsuto S, Yoshida S, Feldmann KA (2001) Overexpression of DWARF4 in the brassinosteroid biosynthetic pathway results in increased vegetative growth and seed yield in *Arabidopsis*. *Plant J* 26:573D582
- Choe S, Schmitz RJ, Fujioka S, Takatsuto S, Lee MO, Yoshida S, Feldmann KA, Tax FE (2002) *Arabidopsis* brassinosteroid-insensitive dwarf12 mutants are semidominant and defective in a glycogen synthase kinase 3beta-like kinase. *Plant Physiol* 130:1506D1515
- Chono M, Honda I, Zeniya H, Yoneyama K, Saisho D, Takeda K, Takatsuto S, Hoshino T, Watanabe Y (2003) A semidwarf phenotype of barley results from a nucleotide substitution in the gene encoding a putative brassinosteroid receptor. *Plant Physiol* 133:1209D1219
- Chuang CF, Meyerowitz EM (2000) Specific and heritable genetic interference by double-stranded RNA in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 97:4985D4990
- Claes B, Dekeyser R, Villarroel R, van den Bulcke M, Bauw G, van Montagu M, Caplan A (1990) Characterization of a rice gene showing organ-specific expression in response to salt stress and drought. *Plant Cell* 2:19D27
- Clouse SD, Sasse JM (1998) Brassinosteroids: Essential regulators of plant growth and development. *Annu Rev Plant Physiol Plant Mol Biol* 49:427D451
- Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in plants. *Plant Physiol* 133:462D469
- Dai Z, Gao J, An K, Lee JM, Edwards GE, An G (1996) Promoter element controlling developmental and environmental regulation of a tobacco ribosomal protein gene L34. *Plant Mol Biol* 32:1055D1065
- Dornelas MC, Lejeune B, Dron M, Kreis M (1998) The *Arabidopsis* SHAGGY-related protein kinase (ASK) gene family: structure, organization and evolution. *Gene* 212:249D257
- Dornelas MC, van Lammeren AA, Kreis M (2000) *Arabidopsis thaliana* SHAGGY-related protein kinases (*AtSK11* and 12) function in perianth and gynoecium development. *Plant J* 21:419D429
- Dubouzet JG, Sakuma Y, Ito Y, Kasuga M, Dubouzet EG, Miura S, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators

- that function in drought-, high-salt- and cold-responsive gene expression. *Plant J* 33:751–763
- Errampalli D, Patton D, Castle L, Mickelson L, Hansen K, Schnall J, Feldmann K, Meinke D (1991) Embryonic lethals and T-DNA insertional mutagenesis in *Arabidopsis*. *Plant Cell* 3:149–157
- Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* 132:6–13
- Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim Biophys Acta* 990:87–92
- Goyal K, Walton LJ, Tunnacliffe A (2005) LEA proteins prevent protein aggregation due to water stress. *Biochem J* 388:151–157
- Jeon JS, Lee S, Jung KH, Jun SH, Jeong DH, Lee J, Kim C, Jang S, Yang K, Nam J, An K, Han MJ, Sung RJ, Choi HS, Yu JH, Choi JH, Cho SY, Cha SS, Kim SI, An G (2000) T-DNA insertional mutagenesis for functional genomics in rice. *Plant J* 22:561–570
- Jeong SW, Choi SM, Lee DS, Ahn SN, Hur Y, Chow WS, Park YI (2002) Differential susceptibility of photosynthesis to light-chilling stress in rice (*Oryza sativa* L.) depends on the capacity for photochemical dissipation of light. *Mol Cells* 13:419–428
- Jonak C, Beisteiner D, Beyerly J, Hirt H (2000) Wound-induced expression and activation of WIG, a novel glycogen synthase kinase 3. *Plant Cell* 12:1467–1475
- Jonak C, Hirt H (2002) Glycogen synthase kinase 3/SHAGGY-like kinases in plants: an emerging family with novel functions. *Trends Plant Sci* 7:457–461
- Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H (1996) Stress signaling in plants: A mitogen-activated protein kinase pathway is activated by cold and drought. *Proc Natl Acad Sci USA* 93:11274–11279
- Kagale S, Divi UK, Kronchko JE, Keller WA, Krishna P (2007) Brassinosteroid confers tolerance to a range of abiotic stresses in *Arabidopsis thaliana* and *Brassica napus*. *Planta* 225:354–364
- Katiyar-Agarwal S, Agarwal M, Grover A (2003) Heat-tolerant basmati rice engineered by over-expression of hsp101. *Plant Mol Biol* 51:677–686
- Kim ST, Kim SG, Hwang DH, Kang SY, Koo SC, Cho MJ, Kang KY (2004) Expression of a salt-induced protein (SALT) in suspension-cultured cells and leaves of rice following exposure to fungal elicitor and phytohormones. *Plant Cell Rep* 23:256–262
- Krishna P (2003) Brassinosteroid-mediated stress responses. *J Plant Growth Regul* 22:289–297
- Kwon M, Choe S (2005) Brassinosteroid biosynthesis and dwarf mutant. *J Plant Biol* 48:1–15
- Lee SC, Kim JY, Kim SH, Lee K, Han SK, Choi HS, Jeong DH, An G (2003a) Trapping and characterization of cold-responsive genes from T-DNA tagging lines in rice. *Plant Sci* 166:69–79
- Lee S, Kim J, Son JS, Nam J, Jeong DH, Lee K, Jang S, Yoo J, Lee J, Lee DY, Kang HG, An G (2003b) Systematic reverse genetic screening of T-DNA tagged genes in rice for functional genomic analyses: MADS-box genes as a test case. *Plant Cell Physiol* 44:1403–1411
- Li J, Chory J (1997) A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. *Cell* 90:825–827
- Li J, Nam KH (2002) Regulation of brassinosteroid signaling by a GSK3/SHAGGY-like kinase. *Science* 295:1299–1301
- Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively. *Arabidopsis Plant Cell* 10:1391–1406
- Mandava NB (1988) Plant growth promoting brassinosteroids. *Annu Rev Plant Physiol Plant Mol Biol* 39:23–52
- McElroy D, Rothernberg MM, Wu R (1990) Structural characterization of a rice actin gene. *Plant Mol Biol* 14:163–171
- Montoya T, Nomura T, Farrar K, Kaneta T, Yokota T, Bishop GJ (2002) Cloning the tomato *Curl3* gene highlights the putative dual role of the leucine-rich repeat receptor kinase tBR11/SR160 in plants steroid hormone and peptide hormone signaling. *Plant Cell* 14:3163–3176
- Nicholas KB, Nicholas HB Jr, Deeb DW II (1997) Gene Doc analysis and visualization of genetic variation. *EMBNEW NEWS* 4:14
- Nomura T, Bishop GJ, Kaneta T, Reid JB, Chory J, Yokota T (2003) The LKA gene is a BRASSINOSTEROID INSENSITIVE 1 homolog of pea. *Plant J* 36:291–300
- Orena SJ, Torchia AJ, Garofalo RS (2000) Inhibition of glycogen-synthase kinase 3 stimulates glycogen synthase and glucose transport by distinct mechanisms in 3T3-L1 adipocytes. *J Biol Chem* 275:15765–15772
- Pay A, Jonak C, Bogre L, Meskiene I, Mairinger T, Szalay A, Heberle-Bors E, Hirt H (1993) The MsK family of alfalfa protein kinase genes encodes homologues of shaggy/glycogen synthase kinase-3 and shows differential expression patterns in plant organs and development. *Plant J* 3:847–856
- Perez-Perez JM, Ponce MR, Micol JL (2002) The *UCR1* *Arabidopsis* gene encodes a SHAGGY/GSK3-like kinase required for cell expansion along the proximodistal axis. *Dev Biol* 242:161–173
- Piao HL, Lim JH, Kim SJ, Cheong GW, Hwang I (2001) Constitutive over-expression of AtGSK1 induces NaCl stress responses in the absence of NaCl stress and results in enhanced NaCl tolerance in *Arabidopsis*. *Plant J* 27:305–314
- Piao HL, Pih KT, Lim JH, Kang SG, Jin JB, Kim SH, Hwang I (1999) An *Arabidopsis* GSK3/shaggy-like gene that complements yeast salt stress-sensitive mutants is induced by NaCl and abscisic acid. *Plant Physiol* 119:1527–1534
- Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Monitoring expression profiles of rice genes under cold, drought, and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. *Plant Physiol* 133:1755–1767
- Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca²⁺-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. *Plant J* 23:319–327
- Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York
- Shinozaki K, Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and cold: differences and cross-talk between two stress signal pathways. *Curr Opin Plant Biol* 3:217–223
- Sun Y, Allen RD (2005) Functional analysis of the BIN 2 genes of cotton. *Mol Genet Genom* 274:51–59
- Sun Y, Fokar M, Asami T, Yoshida S, Allen RD (2004) Characterization of the Brassinosteroid insensitive 1 genes of cotton. *Plant Mol Biol* 54:221–232
- Takakura Y, Ito T, Saito H, Inoue T, Komari T, Kuwata S (2000) Flower-predominant expression of a gene encoding a novel class I chitinase in rice (*Oryza sativa* L.). *Plant Mol Biol* 42:883–897
- Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu Rev Plant Physiol Plant Mol Biol* 50:571–599
- Tichtinsky G, Tavares R, Takvorian A, Schwebel-Dugue N, Twell D, Kreis M (1998) An evolutionary conserved group of plant GSK-

- 3/shaggy-like protein kinase genes preferentially expressed in developing pollen. *Biochim Biophys Acta* 1442:261–273
- Uozu S, Tanaka-Ueguchi M, Kitano H, Hattori K, Matsuoka M (2000) Characterization of XET-related genes of rice. *Plant Physiol* 122:853–859
- Vert G, Chory J (2006) Downstream nuclear events in brassinosteroid signaling. *Nature* 441:96–100
- Yamamoto C, Ihara Y, Wu X, Noguchi T, Fujioka S, Takatsuto S, Ashikari M, Kitano H, Matsuoka M (2000) Loss of function of a rice brassinosteroid insensitive1 homolog prevents internode elongation and bending of the lamina joint. *Plant Cell* 12:1591–1606
- Yang G, Komatsu S (2004) Microarray and proteomic analysis of brassinosteroid and gibberellin-regulated gene and protein expression in rice. *Genom Proteom* 2:77–83
- Yang G, Matsuoka M, Iwasaki Y, Komatsu S (2003) A novel brassinolide enhanced gene identified by cDNA microarray is involved in the growth of rice. *Plant Mol Biol* 52:843–854
- Yoo MJ, Albert VA, Soltis PS, Soltis DE (2006) Phylogenetic diversification of glycogen synthase kinase 3/SHAGGY-like kinase genes in plants. *BMC Plant Biol* 6:3
- Yoshida S, Foro DA, Cock JH, Gomez KA (1976) Laboratory manual for physiological studies of rice. IRRI, Los Baños, Philippines
- Yu J, Hu S, Wang J, Wong GK, Li S, Liu B, Deng Y, Dai L, Zhou Y, Zhang X, Cao M, Liu J, Sun J, Tang J, Chen Y, Huang X, Lin W, Ye C, Tong W, Cong L, Geng J, Han Y, Li L, Li W, Hu G., Huang X, Li W, Li J, Liu Z, Li L, Liu J, Qi Q, Liu J, Li L, Li T, Wang X, Lu H, Wu T, Zhu M, Ni P, Han H, Dong W, Ren X, Feng X, Cui P, Li X, Wang H, Xu X, Zhai W, Xu Z, Zhang J, He S, Zhang J, Xu J, Zhang K, Zheng X, Dong J, Zeng W, Tao L, Ye J, Tan J, Ren X, Chen X, He J, Liu D, Tian W, Tian C, Xia H, Bao Q, Li G, Gao H, Cao T, Wang J, Zhao W, Li P, Chen W, Wang X, Zhang Y, Hu J, Wang J, Liu S, Yang J, Zhang G., Xiong Y, Li Z, Mao L, Zhou C, Zhu Z, Chen R, Hao B, Zheng W, Chen S, Guo W, Li G, Liu S, Tao M, Wang J, Zhu L, Yuan L, Yang H (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. *indica*). *Science* 296:79–92
- Zumbrunn J, Kinoshita K, Hyman AA, Nathke IS (2001) Binding of the adenomatous polyposis coli protein to microtubules increases microtubule stability and is regulated by GSK3 beta phosphorylation. *Curr Biol* 11:44–49